TITLE OF THE INVENTION

Polyphenol oxidase genes from banana, lettuce, tobacco and pineapple

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part application of USSN 08/976,222, filed November, 21, 1997, and International Application No. PCT/AU98/00362 filed May 19, 1998. USSN 08/976,222 claims Paris Convention priority from Australian Patent Application No. PO 6849 filed on May 19, 1997, and is a United States continuation-in-part application of International application No. PCT/AU96/00310 filed on May 22, 1996, which claims Paris Convention priority from Australian Patent Application No. PN 3098, filed May 23, 1995 and Australian Patent Application No. PN 5600, filed September 26, 1995. International Application No. PCT/AU98/00362 claims Paris Convention priority from Australian Patent Application No. PO 6849 filed on May 19, 1997.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not Applicable

FIELD OF THE INVENTION

The present invention relates generally to genetic sequence of plants that encode polyphenol oxidase (PPO) enzymes and functional fragments and parts thereof. More particularly, the present invention provides nucleic acid molecules encoding polyphenol oxidase enzymes of lettuce, banana, tobacco and pineapple plants. The invention further provides methods of isolating said nucleic acid molecules.

BACKGROUND OF THE INVENTION

Browning of plant tissues often occurs following injury or damage and this generally results in spoilage of fruit and vegetables. Undesirable browning also occurs during processing of plant materials to produce food or other products. Steps are taken during transport, storage, and processing to prevent these browning reactions. Often this involves the use of chemicals such as sulphur dioxide but the use of these substances is likely to be restricted in the future due to concerns about their safety and consumer acceptance. For example, the US Food and Drug Administration banned the use of sulphite for most fresh fruit and vegetables in 1986. The production of fruit and vegetable varieties with an inherently low susceptibility to brown would remove the need for these chemical treatments.

It will be understood that browning in plants is predominantly catalysed by the enzyme PPO. PPO is localised in the plastids of plant cells whereas the phenolic substrates of the enzyme are stored in the plant cell vacuole. This compartmentation prevents the browning reaction from occurring unless the plant cells are damaged and the enzyme and its substrates are mixed.

2. Description of Related Art

The prior art includes International Application PCT/AU92/00356 to the present applicant which describes the cloning of PPO genes from grapevine, broad bean leaf, apple fruit and potato tuber. This application recognises that PPO levels in plants may be manipulated by increasing or decreasing expression of PPO gene. The application also identifies two conserved copper binding sites in PPO genes, designated CuA and CuB. However, the method described in PCT/AU92/00356 which was used to clone the PPO genes from apple and potato involved the use of an oligo dT reverse primer for polymerase chain reaction (PCR). Whilst the method is acceptable, in some tissues, it does not give rise to a strong band of the predicted size or else it gives rise to many additional products making it difficult to resolv the PPO fragment.

Accordingly, it is an object of the present invention to overcome or at least alleviate one or more of the difficulties related to the prior art.

SUMMARY OF THE INVENTION

This application is a continuation-in-part application of continuation-in-part application of USSN 08/976,222, filed November, 21, 1997, and International Application No. PCT/AU98/00362 filed May 19, 1998, the entire contents of which are incorporated herein by way of reference.

Bibliographic details of the publications referred to in this specification by author are collected at the end of the description.

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification appear after the claims.

Throughout this specification and the claims that follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

In work leading up to the present invention, the inventors sought to produce improved methods for isolating PPO-encoding nucleic acid molecules which are susceptible for use in modifying the expression of endogenous PPO genes in plants, to reduce browning and modify ripening and storage characteristics of plant tissues and organs.

Accordingly, the inventors have cloned several PPO-encoding g nes from lettuce,

tobacco, banana and pineapple and produced recombinant g ne constructs comprising same for the expression of recombinant PPO polypeptides and nucleic acids capable of modifying the PPO content of plant tissues and cells when expressed therein.

One aspect of the present invention provides an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes or is complementary to a nucleotide sequence which encodes a PPO polypeptide of lettuce, banana, tobacco or pineapple having an amino acid sequence set forth in any one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30, or comprising the copper-binding site of any one of said amino acid sequences.

In an alternative embodiment, the present invention provides an isolated nucleic acid molecule that encodes a PPO polypeptide of lettuce, banana, tobacco or pineapple wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of:

(i) a nucleotide sequence set forth in any one of SEQ ID NOS: 1, 3, 5, 7, 9, 11; 13, 15, 17, 19, 21, 23, 25, 27, or 29;

(ii) a fragment of (i) comprising a nucleotide sequence that encodes the copper-binding site of a PPO polypeptide;

(iii) a degenerate nucleotide sequence of (i) or (ii); and

(iv) a nucleotide sequence that is complementary to (i) or (ii) or (iii).

A second aspect of the invention provides gene constructs comprising the isolated nucleic acid molecules of the invention, preferably in a format suitable for expression in plants, particularly in banana, lettuce, tobacco or pineapples.

A third aspect of the invention provides a method of modifying the endogenous PPO activity of plant cells, tissue, or organs, particularly those cells, tissues, and organs of lettuce, banana, tobacco and pineapples, by expressing the isolated PPO-encoding nucleic acid molecules, or a fragment or anal gue or homologu thereof, in the sense or antisense orientation therein for a time and under conditions sufficient to modify transcription or translation of th ndog nous

mRNA encoding PPO and/or to produce a functional PPO enzym . As used herein, the word "modify" clearly encompasses any alteration to a stated integer, including both a reduction and an increase thereof.

Accordingly, in one embodiment, this aspect of the invention provides a method of increasing the level of lettuce, banana, pineapple or tobacco PPO activity in a plant or a cell, tissue or organ thereof, said method comprising:

(i) introducing a nucleotide sequence to said plant or a cell, tissue or organ thereof which sequence encodes a PPO polypeptide of lettuce, banana, tobacco or pineapple having an amino acid sequence set forth in any one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30, or an enzymatically-active PPO polypeptide comprising the copper-binding site of any one of said amino acid sequences; and

(ii) expressing said nucleotide sequence to produce an enzymatically-active PPO polypeptide.

In an alternative embodiment, this aspect of the invention provides a method of increasing the level of lettuce, banana, pineapple or tobacco PPO activity in a plant or a cell, tissue or organ thereof, said method comprising:

(i) introducing a nucleic acid molecule to said plant or a cell, tissue or organ thereof which nucleic acid molecule comprises the nucleotide sequence set forth in any one of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29, or a degenerate nucleotide sequence thereof; and

(ii) expressing said nucleic acid molecule to produce an enzymatically-active PPO polypeptide.

In an alternative embodiment, this aspect of the invention provides a method of decreasing the level of PPO activity in a plant or a cell, tissue or organ thereof, said method comprising introducing a nucleic acid molecule to said plant or a cell, tissue or organ thereof which comprises a nucleotide sequence selected from the group consisting of:

(i) a nucleotid sequence which encodes a PPO polypeptid of lettuce, banana, tobacco or pineapple having an amino acid s quence set forth in

any one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30, or the copper-binding site of any one of said amino acid sequences;

(ii) a nucleotide sequence set forth in any one of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;

(iii) a fragment of (ii) comprising a nucleotide sequence that encodes the copper-binding site of a PPO polypeptide; and

(iv) a nucleotide sequence that is complementary to (i) or (ii) or (iii).

A fourth aspect of the present invention clearly extends to transfected and transformed cells, tissues, organs and whole organisms that have the subject nucleic acid molecules of the invention introduced thereto. The introduced nucleic acid molecules may exist as extra chromosomal genetic material, or alternatively or in addition, in a form that has been integrated into the cellular genome. This aspect of the invention clearly encompasses transformed plants and plant parts and propagules comprising the subject nucleic acid molecules as an addition to their normal genome composition.

A further aspect of the invention relates to methods of isolating homologues of the nucleic acid molecules exemplified herein, in particular methods relying upon nucleic acid hybridization between highly-conserved regions of the exemplified sequences and nucleotide sequences of homologous PPO-encoding sequences. Such methods include standard nucleic acid hybridizations (i.e. RNA: DNA and RNA: RNA and DNA: DNA) and polymerase chain reaction (PCR)-based and isothermal amplification methods.

According to this aspect of the invention, there is provided a method for preparing nucleic acid encoding an internal fragment of a PPO polypeptide of banana, lettuce, tobacco or pineapple comprising at least a portion of a copper-binding site of said polypeptide or a hybridizable fragment of said nucleic acid, said method including:

(i) providing:

(a) banana, lettuce, tobacco or pineapple PPO cells, tissue or organs having PPO activity;

(b) a first primer having a nucleotide s quence capable of hybridizing to a copper (Cu) binding site-encoding region of a PPO gene or upstream thereof;

(c) a second primer having a nucleotide sequence capable of hybridizing to the complement of a copper (Cu) binding site-encoding region of a PPO gene or downstream thereof; and

(d) an adaptor primer;

(ii) isolating RNA from said cells, tissues or organs;

(iii) treating the RNA to construct copy DNA (cDNA) therefrom; and

(iv) amplifying the cDNA so formed using the first and second primers.

Preferably, the first primer comprises a nucleotide sequence selected from the group consisting of:

(i) 5'-GCGAATTCTT[TC][TC]TICCITT[TC][CA][TC][AC]G-3' (SEQ ID NO: 31);

(ii) 5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3' (SEQ ID NO: 32);

(iii) 5'-GCGAATTCAA[TC]GTIGA[TC][AC]GIATGTGG-3' (SEQ ID NO: 33);

(iv) 5'-GCGAATTCTICA[TC]TG[TC]GCITA[TC]TG-3' (SEQ ID NO: 34);

(v) 5'-GCGAATTCTTICCIT[TA][TC]TGGAA[TC]TGGG-3'(SEQ ID NO: 35); and

(vi) a hybridizable fragment of any one of (i) to (v).

Preferably, the second primer comprises a nucleotide sequence selected from the group consisting of:

(i) 5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3' (SEQ ID NO: 36);

(ii) 5'- GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3' (SEQ ID NO: 37); and

(iii) a hybridizable fragment of (i) or (ii).

Preferably, the treatment of RNA to construct cDNA is performed by treating the RNA with reverse transcriptase and an adaptor primer that comprises the nucleotide sequence:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3' (SEQ ID NO: 38)

or a hybridizable fragment thereof to form cDNA.

Nucleic acid encoding the N-terminal fragment of the PPO polypeptide of banana,

lettuce, tobacco or pineapple can be obtained by attaching an anchor to the 5'-end of the cDNA formed and amplifying said cDNA using a first primer that binds to said anchor and a second primer in the antisense orientation, wherein the nucleotide sequence of said second primer is derived from the sequence of the internal PPO fragment. In this embodiment, the primer in the sense orientation may comprise a nucleotide sequence selected from the group consisting of:

(i)5'-ATATCACCTGTCGGTACATGACGGC-3'(SEQ ID NO: 39);

(ii)5'-GTGCCATTGTAGTCGAGGTCAATCA-3' (SEQ ID NO: 40);

(iii)5'-CCAGTGCCTGGTTTAGGTGTATTCAC-3' (SEQ ID NO: 41); and

(iii) a hybridizable fragment of (i) or (ii) or (iii).

Additionally, in a preferred embodiment, the primer in the antisense orientation may comprise a nucleotide sequence selected from the group consisting of:

(i) 5'TGCTGTTCTGTTCGAACATGGCAG-3' (SEQ ID NO: 42);

(ii) 5'-TATACAAGTGGCACCAGTGTCTGC-3' (SEQ ID NO: 43);

(iii) 5'-CCGCATTGTGGATGACTTCCATCTG-3' (SEQ ID NO: 44);

(iv) 5'-CCAGAATGGGATGGTGAAGGTGTCG-3' (SEQ ID NO: 45); and

(v) a hybridizable fragment of any one of (i) to (iv).

Nucleic acid encoding the C-terminal fragment of the PPO polypeptide of banana, lettuce, tobacco or pineapple can also be obtained by amplifying said cDNA using an adaptor primer and a primer in the sense orientation, wherein the nucleotide sequence of said second primer is derived from the sequence of the internal PPO fragment. In this embodiment, the primer in the sense orientation may comprise a nucleotide sequence selected from the group consisting of:

(i) 5'CGCTGGGTGGGTAATTCTAGGATG-3' (SEQ ID NO: 46);

(ii) 5'-AGTCATCCACAATGCGGCGCACATG-3'(SEQ ID NO: 47); and

(iii) 5'-GTTGCTCTTCTTAGGCTCGGCTTAC-3' (SEQ ID NO: 48)

~~(iv) a hybridizable fragment thereof.~~

The adaptor primer may include the following sequence or a hybridizable fragment thereof: 5'-GACTCGAGTCGACATCG-3' (SEQ ID NO: 49).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation showing the BPPO2 cDNA nucleotide sequence (SEQ ID NO: 1) encoding part of a banana PPO protein, and derived protein sequence therefor (SEQ ID NO: 2).

Figure 2 is a representation showing the BPPO8 cDNA nucleotide sequence (SEQ ID NO:3) encoding part of a banana PPO protein, and derived protein sequence therefor (SEQ ID NO: 4).

Figure 3 is a representation showing the BANPPO34 cDNA nucleotide sequence (SEQ ID NO:5) encoding part of a banana PPO protein, and derived protein sequence therefor (SEQ ID NO: 6).

Figure 4 is a representation showing the BANPPO35 cDNA nucleotide sequence (SEQ ID NO:7) encoding part of a banana PPO protein, and derived protein sequence therefor (SEQ ID NO:8).

Figure 5 is a representation showing the TOBPPO6 cDNA nucleotide sequence (SEQ ID NO: 9) encoding part of a tobacco PPO protein, and derived protein sequence therefor (SEQ ID NO:10).

Figure 6 is a representation showing the TOBPPO25 cDNA nucleotide sequence (SEQ ID NO:11) encoding part of a tobacco PPO protein, and derived protein sequence therefor (SEQ ID NO:12).

Figure 7 is a representation showing the TOBPPO26 cDNA nucleotide sequence (SEQ ID NO:13) encoding part of a tobacco PPO protein, and derived protein sequence therefor (SEQ ID NO:14).

Figure 8 is a representation showing the PINPPO20 cDNA nucleotide sequence (SEQ ID NO:15) encoding part of a pineapple PPO protein, and derived protein

sequence therefor (SEQ ID NO:16).

Figure 9 is a representation showing the PINPPO2 cDNA nucleotide sequence (SEQ ID NO:17) encoding part of a pineapple PPO protein, and derived protein sequence therefor (SEQ ID NO:18).

Figure 10 is a representation showing the PINPPOFL cDNA nucleotide sequence (SEQ ID NO:19) encoding a pineapple PPO protein, and derived protein sequence therefor (SEQ ID NO:20).

Figure 11 is a representation showing the BANPPO1 cDNA nucleotide sequence (SEQ ID NO: 21), and derived protein sequence therefor (SEQ ID NO: 22), including both the putative chloroplast transit sequence and the mature banana PPO protein.

Figure 12 is a representation showing the BANPPO11 cDNA nucleotide sequence (SEQ ID NO: 23) encoding part of a banana PPO protein, and derived protein sequence therefor (SEQ ID NO: 24).

Figure 13 is a representation showing the PINPPO1 cDNA nucleotide sequence (SEQ ID NO: 25) encoding part of a pineapple PPO protein, and derived protein sequence therefor (SEQ ID NO: 26).

Figure 14 is a representation showing the 5PINA cDNA nucleotide sequence (SEQ ID NO: 27) encoding part of a pineapple PPO protein, and derived protein sequence therefor (SEQ ID NO: 28).

Figure 15 is a representation showing the composite LOP1 cDNA nucleotide sequence (SEQ ID NO: 29) encoding a lettuce PPO protein, and derived protein sequence therefor (SEQ ID NO: 30), including both the putative chloroplast transit sequence and the mature banana PPO protein.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a method for preparing nucleic acid encoding PPO, fragments and derivatives thereof, which method includes

providing

a source of a polypeptide having PPO activity,

a first primer having a sequence corresponding to a first conserved region of PPO in sense orientation, and

a second primer having a sequence corresponding to a second conserved region of PPO in antisense orientation;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct copy DNA (cDNA) therefrom; and

amplifying the cDNA so formed using the first and second primers.

Applicant has found that the method of the present invention, which involves the use of a second primer based on PPO, means that there is less likelihood that other (non-PPO) genes are amplified. Furthermore, the method of the present invention dramatically increases the amount of genuine product formed in most cases. Moreover, the added specificity provided by the second PPO-based primer makes it possible to clone PPO more readily from certain plants in which it was difficult to obtain a clone using one primer and oligo-dT. For example, with lettuce cDNA the applicant saw only a faint smear of a range of products with GEN3/GEN8 and oligo-dT but strong bands of the predicted size with GEN3/GEN8 and REV1.

In a preferred aspect of the present invention there is provided a method for preparing nucleic acid encoding banana, lettuce, tobacco or pineapple PPO, fragments and derivatives thereof, which method includes

providing

a source of a polypeptide having banana, lettuce, tobacco or pineapple PPO activity,

a first primer having a sequence corresponding to a first conserved region of banana, lettuce, tobacco or pin apple PPO in sense orientation, and

a second primer having a sequence corresponding to a second

conserved region of banana, lettuce, tobacco or pineapple PPO in antisense orientation;

isolating RNA from th source of polypeptide having banana, lettuce, tobacco or pineapple PPO activity;

treating the RNA to construct copy DNA (cDNA) therefrom; and

amplifying the cDNA so formed using the first and second primers.

The terms "nucleic acid encoding banana/lettuce/tobacco/pineapple PPO" and "banana/lettuce/tobacco/pineapple PPO gene" as used herein should be understood to refer to a banana/lettuce/tobacco/pineapple PPO gene or a sequence substantially homologous therewith. For example, these terms include sequences which differ from the specific sequences given in the Examples hereto but which, because of the degeneracy of the genetic code, encode the same protein. Applicants have found that there are families of PPO genes in most plants. Thus, there are likely to be other PPO genes in lettuce, banana, tobacco and pineapple in addition to those which have been isolated. These could be cloned using the methods of the present invention. Thus, the terms "nucleic acid encoding banana/lettuce/tobacco/pineapple PPO" and "banana/lettuce/tobacco/pineapple PPO gene" should be understood to include banana/lettuce/tobacco/pineapple PPO genes other than those specific genes that have been isolated. The terms may also include presequences such as chloroplast transit sequence as well as sequences encoding mature PPO protein.

The term "derivative" as used herein includes nucleic acids that have been chemically or otherwise modified, for example mutated, or labelled, or nucleic acids incorporating a catalytic cleavage site.

The term "fragment" includes functionally active fragments of a PPO gene which encode a polypeptide or peptide having PPO activity or are capable of altering expression of the PPO genes. Examples of alteration of the gene may include up-regulation or down-regulation of the gene, coding of the gene, transcription of the gene, binding of the gene or stability of the gene sequence.

The source of polypeptide having PPO activity is preferably a source of polypeptide having banana, lettuce, tobacco or pineapple PPO activity. The source of polypeptide having banana PPO activity may be banana fruit, preferably

young banana fruit, more preferably the flesh of young banana fruit. Th source of polypeptid having banana PPO activity may be banana peel, pref rably young banana peel, more preferably the peel of young banana fruit. The source of polypeptide having lettuce PPO activity may be lettuce leaves, preferably young lettuce leaves. The source of polypeptide having tobacco PPO activity may be tobacco leaves, preferably young tobacco leaves. The source of polypeptide having pineapple PPO activity may be pineapple fruit, preferably the flesh of the pineapple fruit, more preferably the flesh of pineapple fruit exhibiting blackheart disorder.

The RNA may be isolated by any suitable method including extraction for example with a detergent such as CTAB, use of an oligo-dT spun column as described in PCT/AU92/00356 the entire disclosure of which is incorporated herein by reference, or use of a commercially available kit such as the PolyATtract 1000 system from Promega Corporation.

The step of treating the RNA to construct cDNA according to this aspect of the present invention may include

treating the RNA with reverse transcriptase and an adapter primer to form cDNA.

The adapter primer may be an oligonucleotide adapter primer including the following sequence or part thereof:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3' (SEQ ID NO: 38)

The step of treating the RNA to construct cDNA according to this aspect of the present invention may include

treating the RNA with reverse transcriptase and reverse primer to form cDNA.

The adapter primer may be replaced with a reverse primer having a sequence corresponding to a conserved region of PPO genes including the following sequence of part thereof:

5'- GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3' (SEQ ID NO: 37)

The first primer has a sequence corresponding to a first conserved region of PPO. Preferably the first primer has a sequence corresponding to at least a portion of or in close proximity to a first copper binding site of PPO. Th second

primer has a sequence corresponding to a second conserved regi n of PPO. Preferably the second primer has a sequence corresponding to at least a portion of or in close proximity to a second copper binding site of PPO. More preferably the first primer has a sequence corresponding to at least a portion of or in close proximity to one of the CuA or CuB binding sites of PPO, and the second primer has a sequence corresponding to at least a portion of or in close proximity to the other of the CuA or CuB binding sites of PPO.

The first and second primers may be degenerate. The first primer may include one of the following sequences or part thereof:

(i) 5'-GCGAATTCTT[TC][TC]TICCITT[TC][CA][TC][AC]G-3' (SEQ ID NO: 31);

(ii) 5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3' (SEQ ID NO: 32);

(iii) 5'-GCGAATTCAA[TC]GTIGA[TC][AC]GIATGTGG-3' (SEQ ID NO: 33);

(iv) 5'-GCGAATTCTICA[TC]TG[TC]GCITA[TC]TG-3' (SEQ ID NO: 34);

(v) 5'-CGCAATTCTTICCIT[TA][TC]TGGAA[TC]TGGG-3'(SEQ ID NO: 35); and

(vi) a hybridizable fragment of any one of (i) to (v).

Preferably, the second primer comprises a nucleotide sequence selected from the group consisting of:

(i) 5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3' (SEQ ID NO: 36);

(ii) 5'- GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3' (SEQ ID NO: 37)

The cDNA may be amplified using the polymerase chain reaction (PCR).

Those skilled in the art will appreciate that if the Cu binding sites are internal, the nucleic acid isolated will be a fragment of the PPO gene lacking 3' and 5' termini. However, it is possible to determine the complete nucleic acid sequence of the PPO gene and to prepare or isolate nucleic acid encoding such PPO or antisense to such PPO.

Accordingly, in a further aspect of the present invention, there is provided a method for preparing nucleic acid encoding the C-terminus of PPO (i.e. comprising the 3'-end of the PPO gene), which method includes:

providing

a source of polypeptide having PPO activity

a primer in sense orientation; and

an adapter primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom; and

amplifying the cDNA so formed using the primers.

There is also provided a method for preparing nucleic acid encoding the N-terminus of PPO (i.e. comprising the 5'-end of the PPO gene), including:

providing

a source of polypeptide having PPO activity,

an anchor,

primers in antisense orientation; and

an anchor primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom;

attaching the anchor to the 5' end of the cDNA so formed; and

amplifying the cDNA using the primers.

The source of polypeptide having PPO activity is preferably a source of polypeptide having banana, lettuce, tobacco or pineapple PPO activity. The source of polypeptide having banana PPO activity may be banana fruit, preferably young banana fruit, more preferably the flesh of young banana fruit. The source of polypeptide having banana PPO activity may be banana peel, preferably young banana peel, more preferably the peel of young banana fruit. The source of polypeptide having lettuce PPO activity may be lettuce leaves, preferably young lettuce leaves. The source of polypeptide having tobacco PPO activity may be tobacco leaves, preferably young tobacco leaves. The source of polypeptide having pineapple PPO activity may be pineapple fruit, preferably the flesh of the pineapple fruit, more preferably the flesh of pineapple fruit exhibiting blackheart disorder.

The RNA may be isolated by any suitable method including extraction for example with a detergent such as CTAB, use of an oligo-dT spun column as described in PCT/AU92/00356 the entire disclosure of which is incorporated herein by reference, or use of a commercially available kit such as the PolyATtract 1000 system from Promega Corporation.

The step of treating the RNA to construct cDNA according to this aspect of

the present invention may include

treating the RNA with reverse transcriptase and an adapter primer to form cDNA.

The adapter primer may be an oligonucleotide adapter primer including the following sequence or part thereof:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3' (SEQ ID NO: 38).

The adapter primer may be replaced with a reverse primer having a sequence corresponding to a conserved region of PPO genes including the following sequence of part thereof:

5'- GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3' (SEQ ID NO: 37)

The primer in sense orientation may be a lettuce PPO specific primer. The primer in sense orientation may include the following sequence or part thereof:

5'CGCTGGGTGGGTAATTCTAGGATG-3' (SEQ ID NO: 46)

The primer in sense orientation may be a banana PPO specific primer. The primer in sense orientation may include the following sequence or part thereof:

5'-AGTCATCCACAATGCGGCGCACATG-3'(SEQ ID NO: 47)

The primer in sense orientation may be a tobacco or pineapple PPO specific primer.

The adapter primer may include the following sequence or part thereof:

5'-GACTCGAGTCGACATCG-3' (SEQ ID NO: 49).

The primers in antisense orientation may be lettuce PPO specific primers. The primers in antisense orientation may include the following sequences or part thereof:

(i) 5'TGCTGTTCTGTTCGAACATGGCAG-3' (SEQ ID NO: 42);

(ii) 5'-TATACAAGTGGCACCAGTGTCTGC-3' (SEQ ID NO; 43)

The primers in antisense orientation may be banana PPO specific primers. The primers in antisense orientation may include the following sequences or part thereof:

(i) 5'-CCGCATTGTGGATGACTTCCATCTG-3' (SEQ ID NO: 44);

(ii) 5'-CCAGAATGGGATGGTGAAGGTGTCG-3' (SEQ ID NO; 45)

The primers in antisense orientation may be tobacco PPO specific primers.

Th primers in antisense orientation may be pineapple PPO specific primers. The primers in antisense orientation may include the following sequences or part thereof:

(i)5'-ATATCACCTGTCGGTACATGACGGC-3'(SEQ ID NO: 39);

(ii)5'-GTGCCATTGTAGTCGAGGTCAATCA-3' (SEQ ID NO: 40)

The anchor may be of any suitable type. The anchor may be attached by ligation for example using T4 RNA ligase. The anchor primer should be capable of hybridizing with the anchor.

The cDNA may be amplified using PCR.

Those skilled in the art will appreciate that using the methods of the present invention it is possible to determine the complete nucleic acid sequence of the PPO gene of interest and to prepare or isolate nucleic acid encoding such PPO or antisense to such PPO.

In a further aspect of the present invention, there is provided a nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig. 1-4, 11 or 12 fragments and derivatives thereof, and substantially homologous sequences.

In a further aspect of the present invention, there is provided a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig.15 fragments and derivatives thereof, and substantially homologous sequences.

In a further aspect of the present invention, there is provided a nucleic acid encoding tobacco PPO or antisense to tobacco PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig. 5, 6, or 7 , fragments and derivatives thereof, and substantially homologous sequences.

In a further aspect of the present invention, there is provided a nucleic acid encoding pineapple PPO or antisense to pineapple PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig. 8-10, 13 or 14 and derivatives thereof, and substantially homologous sequ nces.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for exampl by inclusion of a catalytic cleavage site.

In a further aspect of the present invention there is provided a method for preparing a recombinant vector including a nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof; and

a vector; and

reacting the nucleic acid and the vector to deploy the nucleic acid within the vector.

In a further aspect of the present invention there is provided a method for preparing a recombinant vector including a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof; and

a vector; and

reacting the nucleic acid and the vector to deploy the nucleic acid within the vector.

In a further aspect of the present invention there is provided method for preparing a recombinant vector including a nucleic acid encoding tobacco PPO or antisense to tobacco PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding tobacco PPO or antisense to tobacco PPO, fragments and derivatives thereof; and

a vector; and

r acting the nucleic acid and the vector to d ploy the nucleic acid within the vector.

In a further aspect of the present invention there is provided a m thod for preparing a recombinant vector including a nucleic acid encoding pineapple PPO or antisense to pineapple PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding pineapple PPO or antisense to pineapple PPO, fragments and derivatives thereof; and

a vector; and

reacting the nucleic acid and the vector to deploy the nucleic acid within the vector.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for example by inclusion of a catalytic cleavage site.

The vector may be a plasmid expression vector. For example Bluescript SK$^+$ has been found to be suitable. Alternatively, the vector may be a binary vector. The recombinant vector may contain a promoter, preferably a constitutive promoter upstream of the nucleic acid.

The cloning step may take any suitable form. A preferred form may include

fractionating the cDNA, for example on a column or a gel;

isolating a fragment of the expected size, for example from the column or gel; and

ligating said fragment into a suitable restriction enzyme site of the vector, for example the EcoRV site of a Bluescript SK$^+$ vector.

In order to test the clones so formed, a suitable microorganism may be transformed with the vector, the microorganism cultured and the polypeptide encoded therein expressed. The microorganism may be a strain of Escherichia coli, for example E.coli DH5 has been found to be suitable. Alternatively, appropriate vectors may be used to transform plants.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding banana PPO or antisense to b nana PPO, fragm nts and derivatives th reof, which vector is capable of being

replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding tobacco PPO or antisense to tobacco PPO, fragments and derivatives thereof, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding pineapple PPO or antisense to pineapple PPO, fragments and derivatives thereof, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for example by inclusion of a catalytic cleavage site.

The vector may be a plasmid expression vector. For example Bluescript SK$^+$ has been found to be suitable. Alternatively, the vector may be a binary vector. The recombinant vector may contain a promoter, preferably a constitutive promoter upstream of the nucleic acid encoding banana, lettuce, tobacco or pineapple PPO or antisense to banana, lettuce, tobacco or pineapple PPO, fragments and derivatives thereof.

The microorganism may be a strain of Escherichia coli, for example E.coli DH5 has been found to be suitable.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding banana PPO, a modified nucleic acid encoding banana PPO, or a nucleic acid antisense to banana PPO,

fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding lettuce PPO, a modified nucleic acid encoding lettuce PPO, or a nucleic acid antisense to lettuce PPO, fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding tobacco PPO, a modified nucleic acid encoding tobacco PPO, or a nucleic acid antisense to tobacco PPO, fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding pineapple PPO, a modified nucleic acid encoding pineapple PPO, or a nucleic acid antisense to pineapple PPO, fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

PPO activity may be decreased by the use of sense constructs

(cosuppression). Alternatively the nucleic acid may include a sequ nce encoding antisense mRNA to banana, lettuce, tobacco or pineapple PPO or a functionally active fragment thereof. Alternatively the nucleic acid may encode banana, lettuce, tobacco or pineapple PPO or a functionally active fragment thereof and incorporate a catalytic cleavage site (ribozyme). The nucleic acid may be included in a recombinant vector as hereinbefore described. In a preferred aspect, the nucleic acid may be included in a binary vector. In a further preferred aspect, the introduction of a binary vector into the plant may be by infection of the plant with an Agrobacterium containing the binary vector or by bombardment with nucleic acid coated microprojectiles. Methods for transforming banana, lettuce, tobacco or pineapple with Agrobacterium are known to those skilled in the art and are described in, for example, May et al., Bio/technology (1995) 13:486-492, Michelmore et al., Plant Cell Reports (1987) 6:439-442, and Curtis et al., Journal of Experimental Botany (1994) 45:1141-1149, the entire disclosures of which are incorporated herein by reference.

In a further aspect of the present invention there is provided a method of increasing the level of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding banana PPO or a fragment thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of increasing the level of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding lettuce PPO or a fragment thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of increasing the lev l of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding tobacco PPO or a fragment thereof; and

a plant sampl ; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of increasing the level of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding pineapple PPO or a fragment thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

The nucleic acid may be included in a recombinant vector as hereinbefore described. In a preferred aspect, the nucleic acid may be included in a binary vector. In a further preferred aspect, the introduction of the binary vector into the plant may be by infection of the plant with an Agrobacterium containing the binary vector or by bombardment with nucleic acid coated microprojectiles.

The plant may be of any suitable type. However the method is particularly applicable to banana, lettuce, tobacco or pineapple.

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal banana PPO gene.

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal lettuce PPO gene.

The plant may be of any suitable type. Preferably, the plant is banana.

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal tobacco PPO gene.

~~The plant may be of any suitable type. Preferably, the plant is tobacco.~~

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal pineapple PPO gen .

The plant may be of any suitable type. Preferably, the plant is pineapple.

The nucleic acid may be as hereinbefore described.

EXAMPLE 1

Cloning Lettuce PPO Genes

Messenger RNA (mRNA) was isolated directly from young leaves of lettuce using the PolyATtract 1000 system from Promega Corporation. First strand cDNA was synthesised with reverse transcriptase using a Timesaver cDNA Synthesis Kit (Pharmacia Biotech) utilising an oligo-dT primer adapter as described in Frohman, MA (1990) in "PCR Protocols : A Guide to Methods and Applications" (MA Innis, DH Gelfrand, JJ Sninsky and TJ White, eds) Academic Press, New York pp 28-38, the entire disclosure of which is incorporated herein by reference:

B26 : 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3' (SEQ ID NO: 38)

Oligonucleotide primers were designed based on known plant PPO DNA sequences in the conserved regions of the gene which encode the copper binding sites, CuA and CuB as described in Dry, IB and Robinson, SP (1994) "Molecular cloning and characterisation of grape berry polyphenol oxidase", Plant Molecular Biology 26 : 495-502, the entire disclosure of which is incorporated herein by reference. Two forward primers designed around the CuA site (GEN3 and GEN8) and one reverse primer designed around the CuB site (REV1) were synthesised:

GEN3 : 5'-GCGAATTCTT[TC][TC]TICCITT[TC][CA][TC][AC]G-3' (SEQ ID NO: 31);

GEN8 : 5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3' (SEQ ID NO: 32);

REV1 : 5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3' (SEQ ID NO: 36)

Although the primers are in the region of the Cu binding sites, one of them (GEN8) is just outside of what is traditionally accepted to be a Cu binding site of the enzyme.

The first strand cDNA was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman using GEN3 and REV1 or GEN8 and REV1 primers, each at a final concentration of 1μM (Dry et al.). Amplification involved an initial program of 2 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 2 min, a slow ramp to 72°C over 2 min and elongation at 72°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products and the remainder was purified and concentrated using PCR Wizard Prep columns (Promega Corporation).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E.coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. Three putative lettuce PPO clones (LPO316, LPO812 and LPO813) were identified based on their homology to known plant PPO genes.

Using this sequence information a specific forward primer (LET3P) and two reverse primers (LET5P1 and LET5P2) were synthesised:

LET3P : 5'CGCTGGGTGGGTAATTCTAGGATG-3' (SEQ ID NO: 46);

LET5P1 : 5'TGCTGTTCTGTTCGAACATGGCAG-3' (SEQ ID NO: 42);

LET5P2 : 5'-TATACAAGTGGCACCAGTGTCTGC-3' (SEQ ID NO; 43)

To obtain the 3'-end of the lettuce PPO gene, the first strand cDNA described above was amplified by th same PCR procedure using 1μM LET3P primer and 100 nM adapter primer:

B25 : (5'-GACTCGAGTCGACATCG-3') (SEQ ID NO: 49).

The amplified cDNA was purified as described above and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. A 1000bp fragment was excised from the gel and the DNA was cloned into T-tailed, Eco RV-cut Bluescript SK$^+$ to yield the 3'- end clones LPO9 and LPO10, which were sequenced.

The 5'-end of the lettuce PPO gene was cloned by a modification of the 5'-RACE procedure originally described by Frohman using a 5'-AmpliFINDER RACE kit (Clontech Laboratories). First strand cDNA was synthesised from mRNA with reverse transcriptase using the LET5P2 primer and an AmpliFINDER anchor was ligated onto the 5'-end of the cDNA. The cDNA was amplified by PCR with LET5P1 primer and the AmpliFINDER anchor primer. The amplified cDNA was purified as described above and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. An 850bp fragment was excised from the gel and the DNA was cloned into T-tailed Eco RV-cut Bluescript SK$^+$ to give the 5'-end clones LPO4, LPO5, LPO6, and LPO7, which were sequenced.

The 5'- and 3'-clones were found to have the predicted overlapping sequences with the original clone and the complete sequence of lettuce PPO (LPO1) was derived by combining the sequences from the various clones (Figure 15).

EXAMPLE 2

Cloning Banana PPO Genes

Total RNA was isolated from young banana fruit. Fruit tissue (3g) was frozen and ground to a fine powder in liquid nitrogen with a coffee grinder then added to 20 ml of extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinyl pyrolidone, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.05% spermidine, 2% β-mercaptoethanol) at 65°C. The extract was mixed with 20 ml of chloroform / IAA then centrifuged for 20 minutes at 5,000 RPM and the aqueous phase was re-extracted with chloroform / IAA. The aqueous phase was filtered through Miracloth and 0.25 volumes of 10 M LiCl were added then the sample was incubated overnight at 4°C before centrifuging for 20 minutes at 8,000 RPM. The supernatant was removed and the pellet was resuspended in 0.5 ml f 1 M NaCl, 0.5% SDS, 10 mM Tris, pH 8.0, 1 mM EDTA. The RNA was extracted once with an equal volume of chloroform / IAA and 2 volumes of

ethanol was added. After incubation for 40 mins at -70°C the solution was centrifuged for 15 minutes at 10,000 RPM . The supernatant was removed and the pellet was rinsed with 80% ethanol, drained, and dried. The pellet was resuspended in 50 µl of sterile water.

First strand cDNA was synthesised from 10 µg total RNA with reverse transcriptase as described in Dry, I.B. and Robinson, S.P. (1994) "Molecular cloning and characterisation of grape berry polyphenol oxidase", Plant Molecular Biology 26 : 495-502, the entire disclosure of which is incorporated herein by reference, utilising an oligo-dT primer adapter (Frohman, M.A. (1990) in "PCR Protocols : A Guide to Methods and Applications" (M.A. Innis, D.H. Gelfrand, J.J. Sninsky and T.J. White, eds.) Academic Press, New York pp 28-38, the entire disclosure of which is incorporated herein by reference) :

B26 : 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3' (SEQ ID NO: 38)

Oligonucleotide primers were designed based on known plant PPO DNA sequences in the conserved regions of the gene which encode the copper binding sites, CuA and CuB (Dry et al.). A forward primer designed around the CuA site (GEN3) and a reverse primer designed around the CuB site (REV1) were synthesised :

GEN 3: 5'-GCGAATTCTT[TC][TC]TICCITT[TC][CA][TC][AC]G-3'(SEQ ID NO: 31)

REV1: 5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3' (SEQ ID NO: 36).

The first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman using GEN3 and REV1 primers, each at a final concentration of 1 µM (Dry et al.). Amplification involved an initial program of 2 cycles of denaturation at 94° C for 1 min, annealing at 37° C for 2 min, a slow ramp to 72° C over 2 min and elongation at 72° C for 3 min, followed by 25 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and elongation at 72° C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products and the remainder was purified and concentrated using PCR Wizard Prep columns (Promega Corporation).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA

was introduced into E. coli DH5α by electroporation. R combinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. A putative banana PPO clon (BPO3) was identified based on its homology to known plant PPO genes.

Using this sequence information a specific forward primer (BAN1) and two specific reverse primers (BAN2R and BAN3R) were synthesised:

BAN 1: 5'-AGTCATCCACAATGCGGCGCACATG-3' (SEQ ID NO: 47);

BAN 2R: 5'-CCGCATTGTGGATGACTTCCATCTG-3' (SEQ ID NO: 44); and

BAN 3R: 5'-CCAGAATGGGATGGTGAAGGTGTCG-3' (SEQ ID NO: 45).

To obtain the 3'-end of this banana PPO gene, the first strand cDNA described above was amplified by the same PCR procedure using 1μM BAN1 primer and 100nM adapter primer:

B25: 5'-GACTCGAGTCGACATCG-3' (SEQ ID NO: 49).

The DNA was amplified using 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. The amplified DNA was purified using a QIAquick Spin PCR Purification Kit (QIAGEN) and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. A 1000bp fragment was excised from the gel and the DNA was cloned into T-tailed Eco RV-cut Bluescript SK$^+$ to yield the 3'-end clone BPO17, which was sequenced and shown to encode the 3'-end of BPO3.

The 5'-end of BPO3 was cloned by a modification of the 5'-RACE procedure originally described by Frohmann. First strand cDNA was synthesised from banana fruit RNA as described above but utilising the banana PPO specific primer BAN2R. The DNA was tailed with Terminal transferase as described in Frohmann and amplified by PCR with BAN3R and B26 primers, each at a final concentration of 1μM. The DNA was amplified using 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. The amplified DNA was run on a 1.8% Nusieve GTG (FMC Bioproducts) agarose gel and a 700bp fragment was excised from the gel. The DNA was extracted with a QIAquick Gel Extraction Kit and cloned into T-tailed Eco RV-cut Bluescript SK$^+$ to yield the 5'-end clone BPO26 which was sequenced and shown to encode the 5'-end of BPO3.

The overlapping clones BPO3, BPO17 and BPO26 were fully sequenced in both directions and the sequence of this banana PPO gene (BANPPO1) was derived by combining the sequences (Figure 11).

In the course of these experiments a number of clones were obtained from the banana fruit cDNA by PCR amplification using the B25 primer with one of the degenerate primers based on conserved sequences in other plant PPO genes: GEN7:5'-GCGAATTCAA[TC]GTIGA[TC][AC]GIATGTGG-3' (SEQ ID NO: 33). using the methods described above. Most of these clones were identical to BANPPO1 but one clone, designated BANPPO11, was found to be distinctly different and its sequence is shown in Figure 12.

EXAMPLE 3

Cloning Banana Peel PPO genes

Total RNA was isolated from the peel of young banana fruit. Fruit tissue (3g) was frozen and ground to a fine powder in liquid nitrogen with a coffee grinder then added to 20 ml of extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinyl pyrrolidone, 100 mM Tris-CHl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.05% spermidine, 2% β-mercaptoethanol) at 65°C. The extract was mixed with 20 ml of chloroform / IAA then centrifuged for 20 minutes at 5,000 RPM and the aqueous phase was re-extracted with chloroform / IAA. The aqueous phase was filtered through Miracloth and 0.25 volumes of 10 M LiCl were added then the sample was incubated overnight at 4°C before centrifuging for 20 minutes at 8,000 RPM. The supernatant was removed and the pellet was resuspended in 0.5 ml of 1 M NaCl, 0.5% SDS, 10 mM Tris, pH 8.0, 1mM EDTA. The RNA was extracted once with an equal volume of chloroform / IAA and 2 volumes of ethanol was added. After incubation for 40 mins at -70°C the solution was centrifuged for 15 minutes at 10,000 RPM. The supernatant was removed and the pellet was rinsed with 80% ethanol, drained and dri d. The pellet was resuspended in 50 µL of sterile water.

First strand cDNA was synthesised from 10µg total RNA with reverse transcriptase as described in Ref 2, utilising an oligosaccharide-dT primer adapter (Ref 1):

B26 (SEQ ID NO:38): (5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3').

Oligonucleotide primers were designed based on known plant PPO DNA sequences. Comparison of a number of PPO sequences from a range of different plants allowed identification of the conserved regions of the gene, which are mostly in or near the regions which encode the two copper binding sites, CuA and CuB (2). Forward primers designed around the CuA site (GEN8, GEN9 and GEN 10) and reverse primers designed around the CuB site (REV1 and REV2) were synthesised:

GEN8 (SEQ ID NO: 32): (5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3')

GEN9 (SEQ ID NO: 34): (5'-GCGAATTCTICA[TC]TG[TC]GCITA[TC]TG-3')

GEN10 (SEQ ID NO: 35):(5'-GCGAATTCTTICCIT[TA][TC]TGGAA[TC]TGGG-3')

REV1 (SEQ ID NO: 36):(5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3')

REV2 (SEQ ID NO: 37):(5'-GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3')

The first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman (1) using GEN and REV primers, each at a final concentration of 1µM (2). Amplification involved an initial program of 2 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 2 min, a slow ramp to 72°C for 3 min, followed by 33 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gel and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen).

The purified DNA was cloned into Eco -RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E. coli DH5α by electroporation. Recombinant clones which had an insert of

the predicted size were selected and their DNA sequence was determined by automated sequencing. Two putative banana PPO clones (BPPO2, Figure 1; and BPPO8, Figure 2)were identified by their homology to other plant PPO genes.

The 3'-end of BPPO2 was cloned using a primer designed to the sequence of BPPO2:
BAN8F (SEQ ID NO: 48): (5'-GTTGCTCTTCTTAGGCTCGGCTTAC-3')
at a final concentration of 1μM and a B25 adaptor primer:
B25:(SEQ ID NO: 49): (5'GACTCGAGTCGACATCGA-3')
at a final concentration of 1μM (ref 1). Amplification involved 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gel and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which has been T-tailed with Taq Polymerase and the ligated DNA was introduced into E.coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size (1150 bp) were selected and their DNA sequence was determined by automated sequencing. Two putative banana PPO clones (BANPPO34, Fig. 3; and BANPPO35, Fig. 4) were identified based on their homology to known plant PPO genes. The sequences of BANPPO34 and BPPO2 were identical.

EXAMPLE 4

Cloning Tobacco Leaf PPO genes

Total RNA was isolated from young leaves (1-3cm long) of glasshouse grown plants. Approximately 2 g of frozen leaf material was ground to a fine powder in liquid nitrogen then extracted in 15 ml of extraction buffer (50 mM Tris-HCl, pH 9.0, 150 mM LiCl, 5mM EDTA, 5% SDS and 0.6% β-mercaptoethanol) by shaking vigorously in a 50 ml screw cap tube for 1-2 minutes. Approximately 15 ml of ph nol / chloroform / IAA (25:24:1) was added and th homogenate

mixed then centrifuged for 15 minutes at 5,000 RPM, 4°C. The upper aqueous phase was removed and re-extracted twice with phenol / chloroform / IAA and then once with chloroform / IAA and then centrifuged for 10 minutes at 5,000 RPM, 4°C. The supernatant was removed, LiCl was added to a final concentration of 2 M and the mixture was incubated overnight at 4°C. After centrifuging for 10 minutes at 8,000 RPM, 4°C the supernatant was removed and the pellet was resuspended in 6 ml of 0.4 M LiCl then 2 ml of 8M LiCl was added and the mixture was incubated overnight at 4°C. The mixture was centrifuged for 10 minutes at 8,000 RPM, 4°C, the supernatant was removed and the pellet was resuspended in 0.5 ml of sterile water and centrifuged briefly to remove any insoluble material.

mRNA was isolated from the total RNA using a PolyATtract kit (Promega). First strand cDNA was synthesised from 10 μg total RNA or 2 μg mRNA with reverse transcriptase as described in Ref 2, utilising an oligo-dT primer adapter (Ref 1) :

B26: 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3' (SEQ ID NO: 38).

The first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman (1) using GEN and REV primers described in Example 1, each at a final concentration of 1 μM (2). Amplification involved an initial program of 2 cycles of denaturation at 94° C for 1 min, annealing at 37° C for 2 min, a slow ramp to 72° C over 2 min and elongation at 72° C for 3 min, followed by 28 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and elongation at 72° C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gel and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and th ligated DNA was introduced into E. coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was

determined by automated sequencing. Three putative tobacco PPO clones (TOBPPO6, Fig. 5; TOBPPO25, Fig. 6; and TOBPPO26, Fig. 7) were identified based on their homologies to known PPO genes.

EXAMPLE 5

Cloning Pineapple PPO genes

Mature pineapple fruit were treated to induce blackheart disorder by holding the fruit for 17 days at 12°C then for 4 days at 25°C. Flesh showing blackheart symptoms was dissected from the fruit, frozen in liquid nitrogen and ground to a fine powder in a pre-cooled coffee grinder. To isolate total RNA 10 g of the powder was ground in a mortar and pestle then extracted with 30 ml of homogenisation buffer (100mM Tris-HCl, pH9.0, 200mM NaCl, 15 mM EDTA, 0.5% sarkosyl and 1% β-mercaptoethanol), 30 ml of phenol and 6 ml of chloroform / IAA. The mixture was stirred in a beaker, 2.1 ml of 3M NaAc (pH 5.2) was added and the mixture was kept on ice for 15 minutes then centrifuged for 15 minutes at 8,000 RPM, 4°C. The upper aqueous phase was removed and an equal volume of isopropanol was added. The mixture was incubated for 30 minutes at -70°C then centrifuged for 20 minutes at 8,000 RPM, 4°C in Corex tubes. The supernatant was removed and the pellet was rinsed with 70% ethanol and centrifuged for 5 minutes at 8,000 RPM, 4°C. The ethanol was removed and the pellet was air dried then resuspended in 0.75 ml sterile water and centrifuged to remove any insoluble material. LiCl was added to a final concentration of 3 M and the mixture was incubated overnight at -20°C then centrifuged for 30 minutes at 15,000 RPM, 4°C. The pellet was rinsed with 70% ethanol, centrifuged briefly, drained and air dried. The pellet was resuspended in 75 µl sterile water and centrifuged to remove any insoluble material.

Oligonucleotide primers were designed based on known plant PPO DNA sequences. Comparison of a number of PPO sequences from a range of different plants allowed identification of the conserved regions of the gene, which are mostly in or near the regions which encode the two copper binding sites, CuA and CuB. Forward primers designed around the CuA site (GEN8, GEN9 and

GEN 10) and rev rse primers designed around the CuB site (REV1 and REV2) were synthesised :

GEN8 (SEQ ID NO: 32): (5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3')
GEN9 (SEQ ID NO: 34): (5'-GCGAATTCTICA[TC]TG[TC]GCITA[TC]TG-3')
GEN10 (SEQ ID NO: 35):(5'-GCGAATTCTTICCIT[TA][TC]TGGAA[TC]TGGG-3')

REV1 (SEQ ID NO: 36):(5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3')
REV2 (SEQ ID NO: 37):(5'-GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3')

First strand cDNA was synthesised from 10 μg total RNA with reverse transcriptase as described in Ref 2, utilising the REV2 primer :
REV2 (SEQ ID NO: 37):(5'-GCCTGCAGTT[TC]TC[AG]TC[AG]TAGAA-3')
The first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman (1) using the GEN and REV primers described in Example 1, each at a final concentration of 1 μM (2). Amplification involved an initial program of 2 cycles of denaturation at 94° C for 1 min, annealing at 37° C for 2 min, a slow ramp to 72° C over 2 min and elongation at 72° C for 3 min, followed by 33 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and elongation at 72° C for 3 min.

A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gel and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen). The purified DNA was cloned into Eco RV-cut Bluescript SK^+ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E. coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. A putative pineapple PPO clone (PINPPO20; Fig. 8) was identified based on its homology to known PPO g nes.

First strand cDNA was also synthesised from 10 μg total RNA with reverse transcriptase as described in Dry, I.B. and Robinson, S. P (1994), utilising an oligo-dT primer adapter (Ref 1) :

B26: 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3' (SEQ ID NO: 38).

This first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman, M.A. (1990) using GEN9 and GEN10 primers :

GEN9 (SEQ ID NO: 34): (5'-GCGAATTCTICA[TC]TG[TC]GCITA[TC]TG-3')

GEN10 (SEQ ID NO: 35):(5'-GCGAATTCTTICCIT[TA][TC]TGGAA[TC]TGGG-3')

at a final concentration of 1 μM and a B25 adaptor primer :

B25 : (5'-GACTCGAGTCGACATCGA-3') (SEQ ID NO: 49).

at a final concentration of 0.1 μM (Frohman, M.A. (1990); Dry, I.B. and Robinson, S.P. (1994)) Amplification involved a program of 33 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and elongation at 72° C for 3 min.

A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gel and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E. coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. Two putative pineapple PPO clones (PINPPO1, Fig. 13; and PINPPO2, Fig. 9 were identified based on their homologies to known PPO genes. The sequence of PINPPO1 was nearly identical to that of PINPPO20.

Th 5'-end of PINPPO1 was obtained using a 5'-RACE system for rapid amplificati n of cDNA ends, Version 2.0, from GIBCO-BRL, according to the

manufacturer's instructions. Specific oligonucleotide primers based on the sequences of PINPPO1 and PINPPO2 were used:

PINE 1: (SEQ ID NO: 39): 5-'ATATCACCTGTCGGTACATGACGGC-3'

PINE 2: (SEQ ID NO: 40):5'- GTGCCATTGTAGTCGAGGTCAATCA-3'

A number of clones were sequenced and one, 5PINA (Fig. 14), was found to be nearly identical to PINPPO1 (Fig. 13) in the overlapping regions.

A full-length pineapple cDNA clone was isolated using a primer designed to the 5'-end sequence of 5PINA:

5PIN1: (SEQ ID NO: 41):(5'-CCAGTGCCTGGTTTAGGTGTATTCAC-3')

Primers designed to the 5'-end of the pineapple PPO gene was used with the B25 adaptor primer as described above to amplify cDNA prepared from blackheart-induced pineapple fruit RNA. Amplification involved a program of 33 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min.

A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products. The remainder was run on a low melting point agarose gen and the bands of interest were excised. DNA was purified from the agarose with a QIAquick PCR Purification kit (Qiagen).

The purified DNA was cloned into Eco RV-cut Bluescript SK$^+$ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E.coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size (2.2kbp) were selected and their DNA sequence was determined by automated sequencing. A pineapple PPO clone (PINPPOFL; Fig. 10) was identified based on its homology to the PINPPO20 (Fig. 8), PINPPO1 (Fig. 13) and 5 PINA (Fig. 14) clones. The sequence of PINPPOFL was found to be nearly identical to that of PINPPO20, PINPPO1 and 5PINA in the overlapping regions.

REFERENCES

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